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PRIORITY DOCUMENT

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Applicants: Anne-Marie Rodriguez et al.
U.S. Serial No.: 10/632,581
Filed: July 31, 2003

Exhibit B

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

Docket Number 202819	Type a plus sign (+) inside this box →	+

INVENTOR(S)/APPLICANTS(S)

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TITLE OF THE INVENTION (280 characters max)

ISOLATION OF MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE

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ENCLOSED APPLICATION PARTS (check all that apply)

- ☒ Specification (including any claims and abstract) Number of Pages: 51
- ☐ Drawings Number of Sheets:
- ☐ Power of Attorney
- ☐ Assignment
- ☒ Small Entity Status
- ☐ Other (specify)

METHOD OF PAYMENT (check one)

- ☒ A check is enclosed to cover the Provisional filing fee of $75.00.
- ☐ The Assistant Commissioner is hereby authorized to charge the filing fee of $75.00 to Deposit Account Number 12-1216.
- ☒ The Assistant Commissioner is hereby authorized to charge any fee deficiency or credit any refund to Deposit Account Number 12-1216. A duplicate copy of this communication is enclosed for that purpose.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government:

- ☒ No.
- ☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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☐ Additional inventors are being named on separately numbered sheets attached hereto.

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this correspondence and the documents referred to as attached or enclosed therein are being deposited with the United States Postal Service on October 29, 1999, in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR 1.10, Mailing Label Number EL305735832US, addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Sean Myles Sauer
Printed Name of Person Signing

Signature

PROCVR (Rev. 3/20/96)

PATENT
Attorney Docket No. 202819

Applicant or Patentee: Katz et al.
Serial or Patent No.
Filed or Issued:
For: ISOLATION OF MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS 37 C.F.R. §§ 1.9(f) & 1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: University of Pittsburgh of the Commonwealth System of Higher Education
Address of Organization: 911 William Pitt Union, Pittsburgh, PA 15260

Type of Nonprofit Organization:

☒ University or other institution of higher education.
☐ Tax exempt under Internal Revenue Service Code (26 U.S.C. §§ 501(a) and 501(c)(3)).
☐ Nonprofit scientific or educational organization under statute of state of the United States of America:
Name of State:____________________.
Citation of Statute:____________________.
☐ Would qualify as tax exempt under Internal Revenue Service Code (26 U.S.C. §§ 501(a) and 501(c)(3)) if located in the United States of America.
☐ Would qualify as nonprofit scientific or educational organization under statute of state of the United States of America if located in the United States of America.
Name of State:____________________.
Citation of Statute:____________________.

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled ISOLATION OF MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE, by the inventors Katz et al., described in:

☒ The specification filed herewith.
☐ Application Serial No. , filed .
☐ Patent No. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

In re application of Katz et al.
Atty. Docket 202819

Others Having Rights In The Invention

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d), or a nonprofit organization under 37 C.F.R. § 1.9(e). (NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to his/her/its status as a small entity.)

Name:
Address:

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing:	Frances J. Connell, Esq.
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Oct. 29, 1999
Date

SMALLNPO (Rev. 3/97)

Attorney Docket No.
202819

PATENT APPLICATION

Invention Title:

ISOLATION OF MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE

Inventors:

INVENTOR'S NAME	CITIZENSHIP	CITY OF RESIDENCE	STATE or FOREIGN COUNTRY
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Be it known that the inventors listed above have invented a certain new and useful invention with the title shown above of which the following is a specification.

ISOLATION OF MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE

INTRODUCTION

Clinical Need

Emerging tissue engineering strategies represent a promising and innovative solution to many clinical challenges. This is especially true for many of the soft tissue defects encountered by plastic and reconstructive surgeons. The eventual development of tissue engineered fat equivalents for reconstructive and augmentation purposes will be most welcomed by nearly every surgical discipline and prove to be especially useful for plastic surgeons. The clinical applications for which tissue engineered fat will be particularly useful are vast and varied and can be loosely categorized into *reconstructive*, *cosmetic*, *corrective* and *orthotic* indications.

Reconstructive challenges potentially benefiting from a soft tissue equivalent include: 1) congenital malformations such as hemifacial microsomia and Poland's syndrome, 2) complex traumatic wounds involving soft tissue deficits, 3) pressure sores and 4) oncologic resection defects such as parotidectomies and mastectomies. For perspective, there were over 50,000 breast reconstructions performed in 1997, up 70% from five years ago.[58] From a *cosmetic* perspective, the potential use of engineered soft tissue implants is just as boundless and includes — but is not limited to — 1) augmentation procedures for breast, lips, chin, cheeks, etc., 2) rejuvenation procedures to "fill-out" wrinkles of the aging face and 3) various non-specific revision/sculpting procedures. In 1997, over 120,000 breast augmentation procedures were performed in the U.S. (up nearly 300% from five years ago) and over 60,000 facelifts performed.[58] *Correctional* uses for engineered fat tissue equivalents might include 'disorders' such as stress urinary incontinence (SUI) or vocal cord insufficiency in which a stable, long-lasting "bulking agent" is required. Finally, engineered soft tissue constructs might prove useful for various *orthotic*-related applications in which normally-present soft tissues have atrophied and additional 'padding' or cushioning is needed (such as the ball or heel of the aged foot).

Current Options

Standard approaches to soft tissue reconstruction currently include the use of various local-regional and free microvascular flaps. All of these options are associated to some degree with operative risk, technical difficulty, costly operative time, hospital stay, associated costs and donor site morbidity. Alternative approaches to soft tissue reconstruction and augmentation have traditionally included alloplastic and allogeneic products such as teflon paste, silicone implants and bovine collagen. More recent options include autologous injectable collagen and dermal allograft scaffolds. Each of these methods, however, is associated with certain drawbacks. Alloplastic materials are subject to potential migration and associated with foreign body reactions, allergic reactions and extrusion. Allogeneic materials also include risks of allergic reactions and infection transmission and ultimately fail to integrate into the recipient site for any extended period of time. Newer approaches that involve autologous tissues minimize or overcome issues such as disease transmission and immunogenicity, but still often require multiple and repeated therapies due to implant resorption.

Autologous Fat Transplantation

With the ideal goal being the "replacement of like with like", autologous fat transfer represents a logical approach to soft tissue reconstruction and enhancement. Despite over a century of efforts, however, successful free fat transplantation remains an elusive clinical endeavor. Clinically, most autologous fat transfer is characterized by the gradual but imminent resorption of transplanted fat over time. In the minority of cases in which long-term survival has been documented, issues of predictability and reproducibility remain obstacles that prevent more widespread clinical use. Nevertheless, interest in autologous

free fat transfer has experienced a robust revival in recent years due in part to the widespread popularity of liposuction which both creates defects that need to be "filled" as well as provides a convenient and easy means to obtain tissue for transplantation. In addition, the recent reports of several authors' success with liposuction-based fat transplantation have served to support and encourage the continued pursuit of this somewhat controversial clinical technique.[7, 18] Despite the apparent success of a few, however, the fundamental objectives of *predictability, reproducibility and long-term efficacy* remain significant obstacles to the widespread acceptance of autologous fat transplantation. The emerging discipline of tissue engineering represents an innovative scientific approach to addressing these important issues.

Tissue Engineering Strategies

The future of tissue repair and enhancement will ultimately be enabled by the clinically-guided exploitation of developmental biology. In essence, tissue engineering will soon yield techniques that enable the *(re)*generation of various tissues using cell-based implants fabricated ex vivo as well as strategies that exploit the innate developmental plasticity of a given tissue *in situ*. For example, it is quite likely that many implants of the future will be fabricated using cells isolated from a person's own tissue (harvested via minimally-invasive liposuction techniques). Some of these isolated cells will be banked for future use, and the rest will be grown in culture to increase their numbers. While in culture, the cells may be developmentally manipulated or genetically altered. Ultimately, the cells will be seeded onto a biocompatible polymer scaffold of appropriate size and shape along with extracellular matrix proteins and bioactive factors. The engineered implant will then be implanted back into the patient/cell-donor at the appropriate time. Alternatively, tissue growth and regeneration may be induced via the site-specific delivery of potent bioactive factors that influence the growth and development of *in situ* progenitor/stem cells in a specific manner.{See Figure 1} Although these scenarios seem somewhat futuristic, rapid advances in developmental biology (especially in the identification and purification of extracellular matrix compounds and bioactive factors) have established a solid foundation for achieving these goals. In addition, techniques of cell culture, exogenous cell manipulation and genetic engineering have reached a level of sophistication today that lends palpable credibility to these eventual accomplishments. Plastic surgery and plastic surgeons will undoubtedly play a major role in both the scientific development and clinical utilization of such tissue engineered therapies of tomorrow.

EMERGING TISSUE ENGINEERING STRATEGIES FOR FAT

Compared to other tissues such as skin, bone and cartilage, relatively little effort has been directed toward the tissue engineering of fat. In fact, it is fair to say that most clinicians know relatively little about the development and biology of adipose tissue in general. Yet there is no doubt that significant improvements in fat grafting will require a better understanding of adipose tissue on a cellular and molecular level. Whether adipose tissue is engineered ex vivo as autologous cell-based implants or induced in situ, the basic tenets of tissue transplantation and the fundamental tools of tissue engineering will apply. The common theme of each of these approaches remains the controlled developmental manipulation of adipo-derived cells in a way that strives to *recapitulate normal adipose development*. In other words, the most logical means to generate or *(re)*generate additional adipose tissue for reconstructive or cosmetic purposes is to mimic as best possible the events of natural development. The potential to do so is becoming more and more real as the frontiers of developmental biology explode.

The discussion of adipose tissue engineering that follows is organized into four sections that correspond to the main elements that will be integral to the successful development of autologous, cell-based fat implants. These key elements include: 1)

autologous cell harvest/procurement, 2) cell growth and differentiation, 3) cell implantation and engraftment, and 4) implant integration and histogenesis.

Procurement of Autologous Cells

It is intuitive that one's own (autologous) tissues are the ideal source of cells for tissue engineering. Using cells from another person would require similar immunosuppressive medications necessary for organ transplantation and carry all the expense and risks associated with such. Moreover, critical shortages of most human tissues and organs severely limit the practice of such state-of-the-art medical therapies. Unfortunately, autologous tissues are also hard to obtain. Most cannot be obtained without invasive procedures and/or are not expendable in amounts necessary to make future tissue engineering strategies practical. Fat tissue, however, is the exception. In fact, fat is an ideal source of autologous cells for tissue engineering strategies for several reasons.

First, fat tissue is ***abundant*** in both society and in the average individual. According to a 1995 report by the Institute of Medicine, nearly 60% of the adult population in the United States meets the current definition of clinical obesity.[16] Second, adipose tissue is uniquely ***expendable***. Nearly everyone has enough subcutaneous adipose tissue to donate generous amounts for autologous therapies without any significant biological or anatomical consequences. In fact, a great many people would like nothing better than to 'donate' some of their excess fat tissue! The same is not true for other commonly used sources of autologous tissue such as bone marrow, blood and skin. Third, fat tissue is a source of ***several different cell types*** that may be exploitable for soft tissue regeneration. Adipose tissue is historically perceived as a static collection of engorged, lipid-filled adipocytes that have hypertrophic but not hyperplastic capabilities. In fact, fat is a surprisingly active and dynamic tissue composed of several different cell types including adipocytes, fibroblasts, smooth muscle cells, pericytes, endothelial cells and perhaps most important to tissue engineering, adipogenic progenitor cells, or ***preadipocytes***. Finally, compared to other structural tissues fat tissue is extraordinarily ***easy to obtain*** in large quantities using the minimally-invasive techniques of liposuction. During the two decades since its introduction, liposuction has continually grown in popularity due in part to refined techniques and instrumentation that make it safer and more reliable. Today, it is the most performed aesthetic operation in the world.[58, 62] And while liposuction was originally intended for the removal of unwanted fat tissue and is still predominately utilized as such, it is also an excellent way to harvest autologous tissue for subsequent tissue engineering use.

It was once thought by many that tissue obtained via liposuction was rendered nonviable due to the trauma inherent in the suction process. It has subsequently been shown by our lab and others that this is simply not the case.[37, 39] Our studies confirm the viability of significant populations of mature adipocytes and preadipocytes (as well as other cells in the stromal fraction such as fibroblasts, smooth muscle cells and endothelial cells) in liposuction-harvested human adipose tissue.(manuscript in progress) Even more, we have been able to successfully culture expand such lipo-harvested preadipocytes as well as induce their differentiation in vitro.(See Figure 2 and Figure 3)

Tissue Dissociation

The isolation and culture of specific cells from liposuctioned fat tissue first requires that the "raw" tissue effluent be washed and separated (i.e. dissociated) into its cellular components. Methods currently used for tissue dissociation are tedious, time consuming, labor intensive, subject to contamination and generally limited to the laboratory setting. In addition, liposuctioned tissue presents two unique challenges to this process: **volume and viscosity**. In order to overcome the technical challenges associated with the dissociation of large liposuction volumes, our lab has designed a novel device for the expedient filtration, cleansing and dissociation of large volumes (100ml-1000mls) of human liposuctioned tissue. The ultimate purpose of this device is to make the production-

scale isolation of cells from raw liposuction effluent practical and efficient. A motorized prototype of this device has been fabricated complete with a heat source for optimal enzymatic dissociation.{See Figure 4} The device design allows for the direct transfer of harvested tissue from syringe to device without exposure to air. As a single, self-sufficient unit, it enables the efficient processing (filtration, cleansing and dissociation) of raw liposuctioned tissue in an enclosed, sterile environment. At present, this device is only of use in our laboratory research efforts, but may some day be of use in a clinical or commercial setting.

Cell Growth and Differentiation

Current methods of fat transplantation usually involve small fragments of intact tissue so as to minimize cell death from ischemia. It is thought that single cells may prove even more effective in this setting because they are more likely to survive the initial ischemic period by simple diffusion than pieces of intact tissue, no matter how small. To this end, preadipocytes - which are devoid of lipid and morphologically resemble fibroblasts - are the logical cell candidate on which to base the fabrication of cell-based tissue engineered fat constructs. As alluded to earlier, preadipocytes are now known to exist in the stromal-vascular fraction of adipose tissue along with smooth muscle cells, fibroblasts, endothelial cells and others. Given their smaller size and their lack of intracytoplasmic lipid, preadipocytes seem to be able to tolerate the mechanical trauma and ischemia associated with harvest and implantation better than more fragile mature adipocytes.[42] In addition, under appropriate conditions preadipocytes are able to proliferate and differentiate into mature, lipid-synthesizing and lipid-storing cells both in vitro and in vivo.[17, 45, 54-56] Because of these unique capabilities, it is hypothesized that preadipocyte-based implants will be able to survive implantation well *and* establish an equilibrium of cell turnover that permits optimal host integration and long-term volume (i.e. contour) maintenance.

Preadipocyte Research Models

The great majority of work relating to the cellular and molecular mechanisms of preadipocyte growth and differentiation has been done (and still is) using cell lines or primary cells derived from animals. Only a small amount of work has been done using primary *human cells* derived from subcutaneous depots. Cell lines often used include 3T3-L1, 3T3-F422A, Ob17 and others. In general, cell lines are thought to represent a slightly earlier stage in development than adipogenic precursor cells derived from primary tissues. Cell lines have the advantage of being clonally derived and are therefore a defined, homogenous population of cells presumably at the same stage of differentiation. On the other hand, cultures derived from primary tissues contain cells at varying stages of development. While this is not as scientifically controlled as cell lines, it probably represents a more accurate reflection of the normal in vivo condition.[4]

One of the most common animal sources of preadipocytes is the epididymal fat pad of rats. Despite the widespread use of this source, however, one must wonder what similarities, if any, rat epididymal preadipocytes have to preadipocytes derived from human subcutaneous fat. This is worth consideration for several reasons. First, subcutaneous fat is the most likely source of preadipocytes for tissue engineering strategies for fat. Second, most engineered constructs will ultimately be used for indications that involve placement in subcutaneous sites. And last, as previously mentioned, human fat is unequaled in its availability and is therefore readily obtainable for research purposes. If one intends to work with primary preadipocyte cell populations, it somehow seems inefficient not to take advantage of such a unique tissue supply. The only drawback, of course, is if an animal model is to be used for in vivo studies. In this situation, human cells require the use of an immunocompromised animal model (e.g. athymic mice) to avoid rejection phenomenon. Many would argue that such a model is not an acceptable autologous transplantation model. But is it any more skewed than an autologous model that involves cells from rat epididymal

fat? These issues are endlessly debatable. In the long-run, it will likely be necessary to accrue data in both types of animal models prior to the initiation of human clinical trials.

Preadipocyte Growth

One important factor to consider for ex vivo cell-based implants will be the efficient expansion of preadipocytes in culture. In the 1970s, two different studies demonstrated clear biochemical and developmental differences between the fibroblast-like preadipocytes of fat tissue and fibroblasts of dermis.[45, 54] Since then, much work has been done to delineate the effect of various bioactive factors on preadipocyte growth. Because these various studies have used different cell models or employed different culture methods, direct comparison of results is difficult and sometimes leads to contradictory conclusions. In general, however, factors that have been shown to have a positive effect on preadipocyte proliferation in vitro include the insulin-like growth factor (IGF) family, the fibroblast growth factors (FGF), transforming growth factor β (TGFβ), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and 17β estradiol. Though contradictory results exist, several studies have interestingly shown that preadipocytes from massively obese persons proliferate to a greater extent than do preadipocytes from lean individuals.[38, 47] This observation was further correlated to an increased production of bFGF-related proteins by preadipocytes from obese sources.[50] Obviously, the preadipocyte may play a central role in the pathogenesis of some forms of obesity and this has been written about extensively in the obesity and endocrinology literature. From a tissue engineering standpoint, the study of preadipocytes from pathological conditions may yield valuable new insights into the manipulation of 'normal' cells.

The Adipogenic Lineage

As mentioned before, a thorough understanding of, and ability to control preadipocyte differentiation will be integral to developing techniques for the regeneration of adipose tissue. Embryologically, adipose tissue originates from the mesodermal layer of a developing embryo. Many believe that adipocytes derive from a multipotential stem cell that can also give rise to progenitor cells of bone, muscle, cartilage and other mesodermal tissues.[5] The earliest unipotential cell believed to belong to the adipogenic lineage per se has been termed the *adipoblast* and is thought to derive from mesodermal stem cells. Adipoblasts, in turn, can *commit* to the adipogenic lineage and become preadipocytes. When subjected to a permissible micro-milieu, preadipocytes can undergo differentiation into mature, lipid synthesizing and lipid-storing adipocytes.{See Figure 5} While it is known that preadipocytes at various developmental stages exist within the peripheral fat depots of humans, it remains unclear as to whether mesodermal stem cells and/or adipoblasts exist in peripheral tissues of adults. A more thorough delineation of the developmental cascade summarized above - especially as it pertains to the in vivo condition - is limited by the fact that no stage-specific/cell-specific (e.g. stem cell, adipoblast, preadipocyte) cell-surface markers currently exist allowing for identification and separation of specific subpopulations prior to enzymatic and morphologic changes.

Preadipocyte Differentiation

It is now well established that preadipocyte proliferation and preadipocyte differentiation are inversely related. Based on in vitro work, it seems that preadipocytes must undergo growth arrest (not necessarily contact inhibition) in order to undergo differentiation. Prior to obvious morphological changes (i.e. the accumulation of intracytoplasmic lipid) preadipocytes are 'detected' by the expression of various early markers (LPL, FA transport), intermediate markers (FAS, HSL, aP2, GPAT, ACC and GPDH) and late markers (ACBP, leptin, adipsin).{See Figure 5} Because most serums used in culture medias seem to have a net positive mitogenic effect on cultured

preadipocytes, they also seem to inhibit adipogenic induction. In the late 80s, Deslex et al. reported the use of a serum-free adipogenic media that stimulated increased adipogenic conversion of cultured stromal cell cultures compared to many earlier studies.[10] Subsequent modification of this culture system by Hauner et al. demonstrated that the differentiation of human preadipocytes in vitro is enhanced by the combined presence of glucocorticoids *and* insulin.[21, 24] Adipogenic induction is notably less when either substance is used alone. In addition to insulin and glucocorticoids, elevated levels of cAMP have also been shown to correlate with increased rates of preadipocyte differentiation. On the other hand, factors that seem to have a negative effect on preadipocyte differentiation - either by inhibiting adipogenic induction altogether or by stimulating delipidation of differentiated cells - include TNFα, EGF, TGFβ, endothelin-1 and others.[22, 23, 43, 44, 53]

Not surprisingly, the in vivo control of adipogenesis will prove to be far more complex than in vitro studies suggest. For one, ***all fat is NOT the same.*** There are now documented differences in the growth and differentiation of fat cells derived from different adipose tissue sites.[14, 15, 20, 25] A more recent study demonstrates that human preadipocytes from different sites of the same individual respond differently to a specific adipogenic stimulus on a molecular level.[1] Further work along these lines may elucidate 'optimal' harvest sites for preadipocyte-related tissue engineering goals, if any such sites exist.

Preadipocyte Differentiation: Molecular Control

For the last three decades a good amount of work has been dedicated to the biochemical characterization of preadipocytes including the effects of numerous bioactive proteins and hormones on their growth and differentiation. In order to enable the development of refined techniques for the targeted manipulation of preadipocyte-based implants, however, it is imperative to gain a thorough understanding of preadipocyte growth and differentiation on a molecular level. This, in turn, will eventually result in the ability to engineer the site-specific (re)generation of adipose tissue in a predictable, reproducible and effective manner. To this end, exciting and rapid advances are now being made regarding the molecular mechanisms underlying adipogenic differentiation. Most of these insights have revolved around the detection of adipose-specific genes that become differentially expressed during the adipogenic process. It is now known that tissue-specific gene expression is controlled by transcription factors. Tissue-specific transcription factors are able to influence when and how efficiently a given gene is expressed by binding to specific DNA sites within the enhancer region of that gene. These actions are thought to be mediated by the modulation of histones whereby target genes become exposed for more efficient transcription.[49]

Several transcription factors that play a central role in the control of adipogenesis have now been identified. One that appears to play a prominent role in the regulation of preadipocyte differentiation belongs to the family of *peroxisome proliferator-activated receptors* , or PPARs. PPARs are members of the nuclear receptor subfamily that also includes receptors for the steroid, thyroid, and retinoid hormones. Like other transcription factors, PPARs are able to bind to specific DNA sequences (called PPAR response elements, or PPRE) in the enhancer regions of their target genes. To date, three mammalian PPAR subtypes have been identified : α, β and γ. PPARγ appears to be the most specific and integral to adipogenic differentiation. The PPARγ gene itself contains two promoters that can produce two slightly different PPARγ products, PPARγ1 and PPARγ2. Whereas the former is expressed at low levels in various tissues, PPARγ2 is ***abundantly*** and ***specifically*** expressed in fat tissue.[51, 61] PPARγ2's central role in

adipogenesis is reflected by the fact that while its level is minimal in preadipocytes, its expression increases significantly very early in the adipogenic differentiation process.[52]

Interestingly, PPARγ can only recognize and bind to its targeted DNA sequences (PPREs) when it is combined as a heterodimer with the retinoid X receptor (RXR). Once bound to the response element of a gene's enhancer, PPARγ is able to activate transcription of that gene in the presence of appropriate liganda. In this manner, PPARγ helps to initiate and control adipogenesis by activating the expression of many early genes in the differentiation process. Indeed, its activity is so influential that the forced expression of PPARγ in non-adipocyte cell lines results in adipogenic differentiation.[52]

In general, when nuclear hormone receptors like PPARγ bind to their specific ligands, their DNA binding and transcriptional activities increase significantly. Until recently, however, no stimulatory ligands of PPARγ had been discovered. It is now known that prostaglandins of the D and J series are natural ligands for PPARγ serving to increase its activity, and therefore adipogenesis. For example, 15-deoxy-Δ12,14-PGJ_2 binds directly to PPARγ and promotes efficient adipogenesis in various preadipocyte cell lines.[32] This has paved the way for the search for additional, perhaps better adipogenic stimuli. Some synthetic compounds have already been identified that act specifically through PPARγ. Interestingly, they are all members of the thiazolidinedione family of antidiabetics (TZDs).(for an excellent review, see[49]) Although TZDs act specifically through the PPARγ receptor and adipogenesis is increased by PPARγ liganda in vivo, it remains an unsolved paradox as to why these agents do not result in a significant increase in gross adipose mass/volume in animal models or humans.

Two other transcription factors implicated in adipogenesis are the *CCAAT enhancer binding proteins* (C/EBP) and *adipocyte determination- and differentiation-dependent factor-1/sterol regulatory element-binding protein-1* (ADD-1/SREBP-1). Like PPARγ, the C/EBP family of DNA binding proteins also includes at least three isoforms (α, β and δ). While C/EBPs seem to be necessary for adipogenic differentiation, they alone are not sufficient for such. Recent studies support the notion that C/EBPs β and δ - while not specific to adipose tissue- are involved in adipogenic induction at a stage earlier than the PPARγ receptor. It is now known that the promoter region of the PPARγ gene has at least two C/EBP-binding sites.[61] Furthermore, hormonal induction of C/EBP β and C/EBP δ results in the subsequent expression of PPARγ. C/EBPα, on the other hand, seems to play a role in the maintenance of PPARγ expression. While it is nearly undetectable in preadipocytes, its expression increases notably in the later phases of differentiation. Interestingly, of the three PPAR isoforms, only PPARγ is able to interact with C/EBPα in the induction and maintenance of adipogenic differentiation, and it does so in a synergistic manner.[3]

The role of ADD-1/SREBP-1 in adipocyte differentiation is also starting to emerge. It has been known for some time that ADD-1/SREBP-1 specifically induces the expression of the adipogenic enzymes lipoprotein lipase (LPL) and fatty acid synthetase (FAS). Just recently; however, it has also been shown to increase the transcriptional activity of PPARγ. It does so through the generation of endogenous ligands.[31]

In summary, studies to date suggest that the induction of C/EBP β and δ occurs early in adipocyte differentiation and results in the induction of PPARγ. PPARγ - in the setting

of an appropriate milieu (i.e. ligand) - enables the differentiation process to proceed by activating the efficient transcription of key genes in the developmental cascade. C/EBPα, which is induced relatively later in the adipogenic program maintains and contributes to the terminally differentiated state via its synergistic interactions with PPARγ (and its own direct activation of certain genes).{See Figure 6} A body of knowledge is now emerging that elegantly links the adipogenic effects of various growth factors and hormones - such as insulin and dexamethasone - to the specific transcription factors discussed above.

Implantation and Engraftment

General Considerations

Implantation techniques for preadipocyte-based fat constructs will likely depend on the objectives of an intended use. For example, indications that require only small volume "filler" effects - such as scar revisions, facial augmentation, or urethral obstruction for SUI - might best be served by an injectable delivery vehicle so as to enable minimally-invasive therapy. On the other hand, more conventional implants will likely be necessary for large-volume contour enhancement objectives and complex reconstructive challenges. In general, however, it will be imperative for any engineered cellular construct to be delivered in a manner that optimizes cell survival and initiates and supports cell engraftment. Achieving these goals will most certainly involve the use of extracellular matrix (ECM) factors and/or synthetic biocompatible scaffolds. In addition, the inclusion of various bioactive factors will likely be important for initiating angiogenesis as well as cell turnover and cell differentiation.

Polymer Scaffolds

There are currently several synthetic bioscaffold materials available for testing and use and the list is expanding rapidly. Few of these materials, however, are already FDA approved (such as poly-lactic-co-glycolic acid, or PLGA). The ideal properties of a given scaffold/delivery material would include the following: biocompatibility, biodegradability, amenability to growth factor linkage and delivery, ease of production and ease of use. To date, only a limited amount of work has been done pertaining to biomaterial scaffold support of preadipocytes. Patrick et al. have demonstrated the potential use of PLGA scaffolds for preadipocyte-based implants. More specifically, their work demonstrates that PLGA scaffolds can support the growth and differentiation of rat epididymal preadipocytes both in vitro and in vivo.[41] Others have demonstrated the potential use of calcium alginate gels as a biomatrix for preadipocyte delivery.[46] Obviously, this remains an important and fertile area of investigation, especially as more tissue-specific biomaterials are developed.

Extracellular Matrix

ECM proteins represent both a complimentary and alternative approach to cell support and delivery. It is now well established that the extracellular environment is central to the control of cell growth, migration, differentiation, morphology and function. For instance, an intact basement membrane is essential for the proper function, morphology and differentiation of various cells.[2, 59] The normal basement membrane of white adipose tissue is composed primarily of type IV Collagen and laminin.[19] Interestingly, preadipocytes themselves synthesize various types of collagen (VI, I, III) depending on their stage of differentiation.[9] In fact, at least one study suggests that collagen synthesis is an essential autocrine signal for the terminal differentiation of preadipocytes.[29] It has been postulated that collagen exerts an influence on the expression of late developmental markers through the binding of specific cellular adhesion molecules(e.g. integrins). In a

similar fashion, it is known that certain ECM components act in a paracrine-type setting also. For instance, Varzaneh et al. have demonstrated that ECM components secreted by endothelial cells stimulate preadipocyte differentiation in vitro.[57] Certainly, other similar interactions exist but have not yet been elucidated.

Given the important role of the ECM on cellular development and function, it is logical to exploit such factors in tissue engineering strategies attempting to mimic the natural condition. For example, basement membrane extract from Engelbreth-Holm-Swarm tumor (EHS gel) is a commercially available ECM aggregate that includes both type IV collagen and laminin. EHS gel has been shown to support the in vitro growth of both preadipocytes *and* mature adipocytes.[26, 28, 36] In addition, when combined with bFGF, it is able to induce both angiogenesis and adipogenesis in an animal model.[30, 40] To our knowledge, however, no work has been done evaluating the influence of basement membrane components specifically on *human preadipocyte* growth and differentiation in vitro or in vivo. Our lab is currently engaged in several experiments aimed at evaluating such interactions. Because EHS gel has unique thermosensitive properties - whereby it is a liquid at 4°C but solidifies at body temperature - it can be injected in vivo and therefore is an especially appealing model for minimally-invasive cell delivery.

Neovascularization

The ultimate survival of engineered fat equivalents will depend on the expedient and adequate neovascularization of an implanted construct. Contrary to popular belief, adipose tissue is an exceptionally vascular tissue. This is especially true when one considers that the ratio of endothelial cells to adipocytes is very high due to the bulky nature - and therefore limited number - of adipocytes that can organize around any one given capillary. In addition, it is now clear that adipose tissue is a highly active endocrine tissue that plays a dynamic role in the physiology of energy balance through the secretion of proteins such as tumor necrosis factor alpha (TNFα), leptin, adipsin, plasminogen activator inhibitor, angiotensinogen and probably others that have not yet been identified.

A prominent feature of the histogenesis of fetal adipose tissue is the close spatial and temporal relationship between blood vessel and adipocyte development. This co-dependent relationship is underscored by the historical description of primordial adipose tissue as *primitive fat organs*. While it is agreed that an established blood supply is required for adipose tissue growth, it seems to be a depot dependent matter as to whether vascular development precedes adipocyte development or vice versa.[27] This is especially intriguing given the fact that adipose tissue depots are distinct from one another based on blood vessel morphology and capillary density.[8]

It has long been known that adipose tissue in general is angiogenic.[48] In addition to their proliferative and differentiative capabilites, however, *preadipocytes themselves* have been shown to be angiogenic. This is yet another quality that makes preadipocytes ideal for cell-based approaches to fat tissue engineering. Given the relationship between adipogenesis and angiogenesis during embryonic development, it is not surprising that the maturation of a vascular bed in adipose tissue is tightly connected to both the number and size of differentiating preadipocytes.[6] Several studies have begun to elucidate in more detail the paracrine interactions that exist between preadipocytes and microvascular endothelial cells.[33-35] For example, the novel angiogenic factor monobutyrin (1-butyryl-glycerol) is secreted by differentiating preadipocytes. This compound has been shown to induce endothelial cell proliferation and migration in vitro.[11] Claffey et al. have also demonstrated that vascular endothelial growth factor (VEGF) secretion is upregulated during adipogenesis.[6] VEGF has potent mitotic activity toward endothelial cells. Lastly, several studies implicate bFGF - a known angiogenic and mitogenic growth factor - as a key factor in adipose-related angiogenesis and differentiation.[12, 13, 30]

Implant Integration and Histogenesis

General Considerations

The true mark of success for a tissue engineered therapy will be its ability to seemlessly integrate into the host recipient site for an extended period of time. This objective, in turn, correlates to the technical challenges of predictability, reproducibility and long-term efficacy. After implantation and successful neovascularization, a cell-based construct will need to establish an equilibrium of cell growth and development that matches that of the recipient site. An exact equilibrium of tissue homeostasis will be extraordinarily difficult to achieve, but is a worthy goal nonetheless. Obviously, it is of utmost importance that tissue engineered therapies do not subject a patient to long-term oncologic risks. No matter how thorough and extensive pre-clinical testing is, however, these type of risks may not be evident until years after the clinical introduction of a given therapy.

The concept of host integration refers to a variety of seemingly unconnected factors which culminate in a well-incorporated tissue construct. Cell development and tissue morphogenesis is a highly complex, dynamic process. As mentioned, soluble and insoluble components such as growth factors, inductive factors and biocompatible extracellular matrix materials will undoubtedly be required to control, guide, modulate and support the cellular components of tissue replacements. After implantation of an engineered construct and early cell survival by diffusion and imbibition, subsequent cell survival will depend on angiogenic signals for formal neovascularization. Theoretically, once molecular neovascularization has taken place, surviving progenitor cells in the tissue engineered implant will then be stimulated to replicate and differentiate by the influence of the growth factors and matrix components included in the implant as well as the local micro-milieu. As the matrix scaffold degrades over time, the differentiating cells will secrete their own matrix components resulting in the formation of a "neo-tissue". With more time this will continue to remodel itself into mature tissue.

Growth Factor Delivery

Based on the scenario presented above, it is evident that bioactive factors will play a key role in the integration and morphogenesis of a tissue engineered implant. As mentioned, bioactive factors can influence a wide variety of activities including cell motility, cell-ECM interactions, cell growth and cell development. In order to achieve desired effects, however, it is now becoming clear that bioactive factors will need to be delivered: 1) over extended periods of time, 2) in variable concentration gradients, 3) with specific chronicity and/or 4) within certain 'response windows' of opportunity. This type of controlled delivery presents substantial challenges to tissue engineers. Because many bioactive factors have extremely short half-lives, in vivo delivery in simple solution is often not effective. There are, however, several emerging alternative strategies to bioactive factor delivery that show good promise. These approaches include: 1) delivery via incorporation directly into biomatrix scaffolds and/or ECM components, 2) delivery via polymer microspheres and 3) delivery via genetic engineering/gene therapy techniques. For example, at least two groups have demonstrated the potential use of basement membrane components for the delivery of angiogenic and mitogenic growth factors.[30, 40] In addition, Yuksel et al. have initiated early work that suggests long-term delivery of growth factors in vivo is feasible using PLGA/PEG microspheres.[60]

Our lab has completed proof-of-concept studies that suggest preadipocytes themselves can be genetically modified to deliver a given protein/growth factor. More specifically, we have successfully transduced human lipo-derived stromal cells (preadipocytes) ex vivo with three different viral vectors (retrovirus, HSV and adenovirus) to express one of two different marker genes (LacZ and Green Fluorescent Protein (GFP)). [See Figure 7] We also have preliminary data suggesting excellent in vitro secretion of bFGF from genetically engineered human lipo-derived cells. Human lipo-derived cells were transduced in vitro

with an HSV-bFGF vector. Levels of bFGF in culture supernatants were then measured at various timepoints using an ELISA (enzyme-linked immunosorbent assay). The results demonstrated elevated (though transient) secretion of bFGF from genetically engineered cells compared to non-transduced control cells with a peak at 3 days post-transduction. Though intriguing, these results need to be repeated and then followed up in an in vivo model. Along these lines, we are now exploring the in vivo survival and growth of human lipo-derived cells genetically-modified with a marker protein.

Despite the early nature of most of these studies, it does seem reasonable to conclude that human lipo-derived stromal cells are amenable to ex vivo genetic modification using viral vectors. As technologies for vector construction and gene therapy continue to evolve, the abundant and readily-available nature of human lipo-derived cells makes them a potentially useful, appealing and *practical* delivery 'vehicle' for a variety of autologous gene therapy strategies.

CONCLUDING REMARKS

The field of tissue engineering strives to integrate technologies from multiple, seemingly unrelated disciplines for the purpose of generating innovative and revolutionary therapies for tissue repair and replacement. A huge clinical need exists for tissue engineered fat equivalents, ranging from formal reconstructive applications to various cosmetic/augmentation procedures. As new insights into the molecular aspects of adipose development accrue, autologous cell-based tissue engineering strategies are beginning to emerge. These approaches will likely involve some permutation of autologous preadipocytes, biocompatible polymer scaffolds, extracellular matrix components and bioactive factors that result in the ex vivo fabrication of adipose tissue implants. Preadipoycytes are an ideal foundation for cell-based fat equivalents because they are hardy and they possess angiogenic, proliferative and adipogenic potential. The necessary knowledge and tools for the development of *'first generation'* adipose tissue constructs (e.g. small volume "fillers") that require only molecular neovascularization for survival are at hand. For example, techniques for autologous tissue harvest and high-volume preadipocyte isolation currently exist and are continuously being refined. Techniques for preadipocyte culture, expansion, differentiation and genetic modification also currently exist and they too continue to be refined as molecular transcription factors and similar compounds become identified and characterized. Nevertheless, the introduction of engineered cell constructs to the clinical realm is still a few years away.

The development of larger, more complex *'second generation'* constructs (e.g. breast implants, composite tissues) that will require defined vascular systems for neovascularization are even further away from clinical reality. These types of implants will only evolve as other co-enabling technologies become refined and practical (such as tissue engineered blood vessels, computerized assembly/fabrication techniques for the cell-seeding of complex, 3-dimensional scaffolds and "bioreactors" that can sustain such constructs ex vivo prior to implantation).

All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims appended hereto.

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Text

Introduction: Little is known about the cellular mechanisms of free fat transplantation. Though metabolically active, mature adipocytes are fragile lipid-laden cells that do not withstand the mechanical trauma of suction-harvest and syringe injection well. What is more, the triglycerides and free fatty acids that are released from damaged adipocytes are damaging to surrounding cell membranes, thus setting up a 'vicious cycle' of cell death. In the past, adipocyte stem cells, or preadipocytes, have been implicated in the development of adipose tissue and more recently have been postulated to play a major role in free fat grafting. Preadipocytes are non-lipid containing cells that exist within the stromal portion of adipose tissue. They are able to proliferate as well as stimulate angiogenesis. In addition, they are capable of developing into mature adipocytes given the appropriate environment. To date, however, no one has actually demonstrated whether or not preadipocytes exist within primary human liposuction effluent. Obviously, this has major implications for clinical fat transplantation efforts since liposuction plays a central role in most free fat grafting techniques. Liposuction provides a quick, safe and efficient means to harvest large amounts of autologous tissue/cells for transplantation. As such, the purpose of this study was to determine if viable preadipocytes exist within human liposuctioned fat tissue.

Methods: Human adipose tissue was obtained from routine elective liposuction procedures. Raw liposuction effluent was strained so as to separate adipose tissue pieces from associated liquid waste. Isolated tissue was rinsed thoroughly and then enzymatically dissociated. Stromal cells were then isolated by way of centrifugation and filtration. The isolated stromal cells were plated at a density of 30 - 50 cells/cm2 in 10% serum containing media for 24 hours. After rinsing with buffer, the cells were then grown in a serum-free media reported to induce adipocyte development (i.e. adipogenesis). Cells were then observed for morphological changes consistent with adipocyte differentiation (e.g. intracytoplasmic lipid accumulation) and stained with Oil Red O to confirm the presence of triglycerides.

Results: Viable stromal cells were successfully isolated from human liposuctioned fat tissue. Adherent cultures of such cells revealed a heterogeneous cell population with predominantly fibroblast-like cells. After 2-3 weeks exposure to serum-free adipogenic media, variable proportions of cultured stromal cells accumulated intracellular lipid that stained positively with Oil Red O, thus confirming the de novo synthesis of triglycerides. As adipocyte development progressed, intracellular lipid collections became larger and unilocular as the cells themselves attained a more rounded shape. Ultimately, fully differentiated adipocytes lifted from the culture surface due to their increased buoyancy.

Conclusion: This study demonstrates for the first time that viable adipocyte stem cells exist within primary ***human liposuctioned*** fat tissue effluent. Moreover, we have shown that such liposuction-procured cells can be cultured and maintain the ability to develop into mature adipocytes under certain conditions. These finding have direct implications on autologous fat grafting strategies. Because preadipocytes have proliferative potential as well as developmental potential and because they are capable of mediating angiogenesis and neovascularization through paracrine interactions with endothelial cells, they most likely play an integral role in free fat transplantation. This work provides an excellent foundation on which to base further research into the cellular mechanisms of free fat transplantation, the healing response of fat and fat regeneration/development in general.

CULTURE AND DIFFERENTIATION OF PREADIPOCYTES ISOLATED FROM HUMAN LIPOSUCTIONED ADIPOSE TISSUE

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Introduction: Adipocyte progenitor cells, or preadipocytes, are fibroblast-like cells devoid of lipid that exist within the stromal fraction of adipose tissue. They are capable of self-renewal, angiogenic signaling and terminal differentiation - ideal qualities for cell-based tissue engineering efforts. Liposuction potentially provides a quick, safe and efficient means to harvest large amounts of autologous tissue/cells for transplantation, gene therapy or tissue engineering applications. We hypothesized that viable preadipocytes could be isolated from human liposuctioned fat tissue, cultured and terminally differentiated in vitro. **Methods:** Human subcutaneous adipose tissue was obtained from elective liposuction specimens (n=10) and preadipocytes isolated with a novel device via an enzymatic dissociation, centrifugation and filtration process. Trypan blue was used to determine cell viability and primary cells were plated at a density of 100,000 cells/cm2 in 10% serum-containing media. At confluence, the media was changed to a serum-free 'adipogenic media' containing dexamethasone (100nM) and insulin (10µg/ml). Cultures were observed daily for the accumulation of intracytoplasmic lipid and subsequently stained with Oil Red O to confirm the presence of lipid. **Results:** Immediately following the dissociation process, cell viability ranged from 50-75%. Cultures revealed a heterogeneous cell population with fibroblast-like cells that grew to confluence and were amenable to repeated subculture. Exposure to adipogenic media induced a subpopulation of stromal cells to accumulate intracellular lipid that stained positively with Oil Red O, a finding consistent with the terminal differentiation of preadipocytes. **Conclusions:** We conclude that: 1) viable preadipocytes exist within, and can be isolated from primary human liposuctioned fat tissue and (2) that such cells can be expanded and terminally differentiated in-vitro. This study represents the first such evidence that liposuction is a source of viable preadipocytes that may be useful for a variety of clinical applications.

LIPOSUCTIONED FAT: A POTENTIAL SOURCE OF AUTOLOGOUS CELLS FOR GENE THERAPY APPLICATIONS

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Introduction: Many ex-vivo gene therapy strategies are limited by the availability of autologous cells for gene delivery. Adipose tissue, however, would be an ideal source of autologous cells for such gene therapy strategies because of its abundance, expendability and ease of procurement. We hypothesized that cells isolated from human liposuction-procured adipose tissue could express a genetically engineered protein in-vivo following ex-vivo culture-expansion, transduction and implantation. **Methods:** Human adipose tissue was obtained from elective liposuction procedures. Stromal cells were isolated by a novel enzymatic dissociation, centrifugation and filtration technique. Cells were culture-expanded in 10% serum-containing media with 10ng/ml bFGF and transduced with a non-replicating adenoviral-green fluorescent protein (GFP) gene construct (MOI=100) after the third passage. 48hrs. after transduction, implants (n=8) consisting of 100µl matrigel™, 100ng/ml bFGF and transduced lipo-derived stromal cells (8x10^6/ml) were injected subcutaneously into nude mice using a 1cc syringe and a 27 gauge needle. Acellular implants consisting of matrigel™ and bFGF served as controls. At 2 and 6 weeks post-implantation, animals were sacrificed and implants harvested. Implants were then cryostat-sectioned and examined for protein expression/cell survival using fluorescent microscopy. **Results:** Fluorescent microscopy of transduced cells prior to implantation revealed excellent gene uptake and GFP expression. Subsequent evaluation of cell-containing implants at two weeks post-implantation also revealed GFP expression, while acellular control implants did not. Implants at six weeks revealed no GFP expression in either group. **Conclusions:** We conclude that: 1) primary human lipo-derived stromal cells can be efficiently transduced ex-vivo with an adenoviral vector and can transiently express a fluorescent marker protein (GFP) in-vivo after implantation; 2) the transient protein expression observed in this study is consistent with the nature of adenoviral-mediated gene transfer; and 3) adipose tissue may be a valuable and plentiful source of autologous cells for gene therapy applications.

BASIC FIBROBLAST GROWTH FACTOR GENE THERAPY USING HUMAN LIPO-DERIVED CELLS

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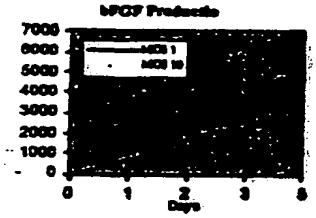
Introduction: Basic fibroblast growth factor (bFGF) is a potent mitogen that potentiates angiogenesis. Ex vivo gene therapy is a promising strategy for the site-specific delivery of bFGF to wounds and recipient graft beds. Human lipo-derived adipose tissue is an abundant and easily harvested source of cells for gene therapy. The purpose of this study was to determine if cells from human lipo-derived adipose tissue could be transduced with the bFGF gene. **Methods:** Human lipo-derived stromal cells were isolated via collagenase dissociation. Cells were cultured and divided into four groups. At confluence two of the groups were transduced with a bFGF-herpes simplex virus (HSV). The other two groups served as controls. Supernatant samples were collected at 1, 2, 3, and 5-day intervals and analyzed via an ELISA for bFGF. **Results:** ELISA analysis revealed suprabasal expression of bFGF in experimental cultures. At a multiplicity of infection (MOI) of 10, analysis of variance (ANOVA) revealed a statistically significant difference ($p<0.0001$) in bFGF levels between the serially collected 24 hour samples. Days 2 and 3 were compared to days 1 and 5 and to each other and found to be significantly different in all cases ($p<0.05$). When a MOI of 10 and a MOI of 1 were compared with each other and against the noninfected control, ANOVA showed a statistically significant difference on day 3. Further comparisons of day 3 data showed a statistically significant difference between all three groups.



Conclusions: This study demonstrates that cultured human lipo-derived stromal cells can be transduced with nonreplicating bFGF-HSV. Suprabasal levels of bFGF are detectable 48 to 72 hours post-transduction. Peak levels are reached at 72 hours and increasing the multiplicity of infection can augment levels. As such, lipo-derived adipose tissue represents an appealing source of autologous cells for the potential site-specific delivery of bFGF and other bioactive factors.

Adipogenic Stem Cells (Preadipocytes) from Liposuctioned Adipose Tissue and Uses Thereof

AJ Katz et al.

The quest for the generation of new tissues and/or tissue equivalents for lost or damaged tissues and organs is now well underway. Many of the strategies underlying these tissue engineering efforts involve autologous cell transplantation paradigms. The general idea of this approach is to: 1) harvest tissue from a patient, 2) isolate undeveloped stem/progenitor cells, 3) expand and developmentally/genetically manipulate such cells in culture, 4) combine such cells with bioactive factors, extracellular matrix proteins, and/or biodegradable polymer scaffolds, and then 5) re-implant/deliver the resulting 'neo-tissue' back into the original donor-patient for a specific therapeutic purpose.[See diagram #1] The ultimate goal of this strategy is to exploit the growth and developmental potential of a particular stem/progenitor cell population in a manner that recapitulates normal development as close as possible.

Great advances have been made recently in the engineering of tissues such as skin, bone, and cartilage. In contrast, minimal attention and effort has been directed toward the fabrication of autologous adipose tissue (i.e. fat). At first glance, it might seem undesirable to grow more fat tissue for someone; but in fact, an engineered fat implant would be of great benefit to many patients and surgeons. Such technology would prove invaluable in addressing markets/clinical challenges as diverse as: 1) traumatic tissue loss, 2) oncologic-related tissue resections (e.g. mastectomy), 3) congenital tissue deficiencies, 4) tissue loss secondary to aging, 5) facial wrinkles and other cosmetic-related indications, 6) stess urinary incontinence, and 7) vocal cord paralysis.

Standard approaches to soft tissue reconstruction currently include the use of various local-regional and free microvascular flaps. All of these options are associated to some degree with operative risk, technical difficulty, costly operative time, hospital stay, associated costs and donor site morbidity. Alternative approaches to soft tissue reconstruction and augmentation have traditionally included alloplastic and allogeneic products such as teflon paste, silicone implants and bovine collagen. More recent options include autologous injectable collagen and dermal allograft scaffolds. Each of these methods, however, is associated with certain drawbacks. Alloplastic materials are subject to potential migration and are associated with foreign body reactions, allergic reactions and extrusion. Allogeneic materials also include risks of allergic reactions and infection transmission and ultimately fail to integrate into the recipient site for any extended period of time. Newer approaches that involve autologous tissues minimize or overcome issues such as disease transmission and immunogenicity, but still often require multiple and repeated therapies due to implant resorption.

With the ideal goal being the "replacement of like with like", autologous fat transfer represents a logical approach to soft tissue reconstruction and enhancement. Currently this involves tissue harvest via liposuction, then tissue "preparation" followed by immediate re-injection of the free tissue into a new site. Despite over a century of efforts, however, free fat transplantation remains an imperfect endeavor. Clinically, most autologous fat transfer is characterized by the gradual but imminent resorption of transplanted fat over time. In the minority of cases in which long-term survival has been documented, issues of predictability and reproducibility remain obstacles that prevent more widespread clinical use. Nevertheless, interest in autologous free fat transfer has experienced a robust revival in recent years due in part to the widespread popularity of liposuction which both creates defects that need to be "filled" as well as provides a convenient and easy means to obtain tissue for transplantation. In addition, the recent reports of several authors' success with liposuction-based fat transplantation have served to support and encourage the continued pursuit of this somewhat controversial clinical technique. In 1996, there were approximately 13,000 autologous fat transplantation procedures performed by plastic surgeons in the U.S. This number has almost certainly grown over the last two years due to: 1) the appeal of using one's own tissues for treatment (thereby avoiding infectious, allergic and rejection risks), 2) the allure of "putting excess fat to good use", and 3) increased media coverage in both the professional and lay press. Despite the apparent success of a few, however, the fundamental objectives of predictability, reproducibility and long-term efficacy remain significant obstacles to the widespread acceptance of autologous fat transplantation.

The emerging discipline of tissue engineering represents an innovative scientific approach to resolving these important issues.

From an autologous tissue engineering perspective, fat tissue is unmatched by any other tissue in regards to availability, expendability and ease of procurement. First, fat tissue is extraordinarily abundant on a societal as well as individual level. Second, fat tissue is uniquely expendable compared to any other tissue (including skin and blood) with no known physiologic or anatomic morbidities associated with removal of exploitable quantities. And third, using liposuction techniques, it is unusually easy to obtain (harvest) adipose tissue in a safe, cosmetically-pleasing, minimally-invasive, outpatient manner.

Fat tissue is known to contain undifferentiated progenitor (stem) cells referred to by many as preadipocytes. Preadipocytes are fibroblast-like cells that exist within the stromal fraction of adipose tissue and have the potential to differentiate, or develop, into mature lipid-bearing adipocyte cells. In addition, they are able to proliferate and secrete angiogenic substances (which stimulate the growth of new blood vessels). All of these qualities make preadipocytes a prime candidate cell on which to base tissue engineering strategies. In conjunction with polymer scaffolds, extracellular matrix factors and bioactive factors, it is believed that preadipocytes will be able to direct both the ex-vivo generation of adipose tissue equivalents as well as site-specific re-generation in-situ.

Although preadipocytes have been described and isolated from adipose tissue in the past, strategies for the production-scale isolation of autologous human preadipocytes from liposuction-harvested tissue have yet been described. Liposuction - introduced nearly 20 years ago - represents an ideal method of fat tissue harvest for large-scale cell isolation due to its safe, minimally-invasive nature and potential for body-image improvement. Today liposuction is the most performed aesthetic operation in the world. It was long thought that liposuction was too traumatic a procedure to yield any significant amounts of viable tissue/cells, especially given the historically poor results of free autologous fat transfer. As such, while preadipocytes are known to exist within adipose tissue, it has never been demonstrated that they exist within liposuction effluent; and if so, that they can be successfully isolated from such for culture and further manipulation.

Given the advancement of tissue engineering over the last few years, the ability to isolate, culture and developmentally manipulate preadipocytes (and other cells) from primary human liposuctioned fat tissue would provide an excellent clinical and commercial foundation in the burgeoning area of soft tissue (fat) engineering. Using a modification of existing techniques in conjunction with our own invention, our lab has demonstrated the ability to isolate, culture, and differentiate human preadipocytes from large volumes of primary human liposuctioned fat tissue on a routine basis. This enabling technology holds worthwhile commercial potential given the rapidly evolving fields of tissue engineering and gene therapy.

Methods:

Human subcutaneous adipose tissue is obtained from elective liposuction procedures with appropriate consent. The raw liposuction effluent (ranging from 100 - 5000mls) is strained in our proprietary device (U.S. patent # 5,786,207) or layered in a modified media bottle to 'refine' (isolate and wash) the actual pieces of adipose tissue from the associated liquid waste consisting of oil, serum, blood, anesthetic solution, etc. The refined fraction of tissue is then subjected to an enzymatic dissociation and filtration process to create a single cell suspension. Stromal cells are separated into a pellet by centrifugation and washed several times by repeated centrifugation and resuspension. Red blood cells are lysed and the remaining cell suspension filtered to remove debris and cell 'clumps'. Cell number and viability are determined with trypan blue dye exclusion and a hemacytometer.

Stromal cells are plated onto standard culture flasks at densities ranging from 20,000 - 100,000 cells/cm2 and grown in DMEM/Ham's F12 media (1:1) with 10% fetal bovine serum and penicillin/streptomycin until they reach confluence. At confluence, cultures are washed 2-3 times with PBS and then grown in a serum-free adipogenic media consisting of:

DMEM/Ham's F12 (1:1) 1000mls
1M NaHCO3 (15mls)
Biotin (33uM)
Pantothenate (17uM)
Transferrin (10ug/ml)
Dexamethasone (100nM)
Insulin (10ug/ml)
T3 (0.2 nM)
Pen/Strep

Media is changed every 2-3 days and the cyclic-AMP enhancer isobutyl-methylxanthine (IBMX) is added to the first feed to accentuate adipogenic induction.

After induction with adipogenic media, cells are observed daily with phase-contrast microscopy to identify morphological changes associated with the terminal differentiation of preadipocytes: rounding and the accumulation of intracytoplasmic lipid droplets. Oil Red O stain is used to confirm the presence of lipid and hematoxylin or hoescht's stain is used to identify cell nuclei. After staining, digital images can be acquired and the images subsequently analyzed with software to quantitate the amount of Oil Red O - positive lipid and the number of cells per a given area.

Results:

Stromal cells can be isolated from 'raw', primary human liposuctioned fat tissue with viabilities in the range of 40 - 80%. On average, approximately 100,000 viable cells can be isolated from the dissociation of each 1ml of refined lipo-procured adipose tissue. In primary culture, the stromal cells appear as a heterogeneous population with a predominant fibroblast-like appearance.[Figures 1 and 2] After attaining confluence they can be subcultured (passaged) multiple times using standard typsinization methods. After adipogenic induction, a varying percentage of stromal cells undergo terminal differentiation over a 1-4 week time period. Such adipogenic conversion is characterized by the de novo synthesis of lipid and its accumulation intracellularly.[Figure 3] Positive Oil Red O staining confirms the presence of lipid.[Figure 4] Initially, lipid droplets are small and multiple. Gradually, however, they begin to fuse into fewer but larger droplets. As this occurs, the cell shape changes from fusiform to round. The combination of a more rounded shape and increasing buoyancy from intracellular lipid causes differentiating cells to eventually lift-off from the culture surface and float up into the media.[Figures 5-8] At this point, it is difficult to follow and maintain the cells.

The earliest morpholocial signs of differentiation (i.e. intracellular lipid accumulation) occurs around 7 days after induction, but the majority of conversion is seen 2-3 weeks after induction. Depending on a multitude of variables that includes, but is not limited to donor age, site, sex, weight and harvest method, varying proportions of stromal cell cultures terminally differentiate after induction. Subjectively, the conversion rate ranges between 30- 80%, with a mean of approximately 60%. In addition, the addition of IBMX with each media change seems to enhance adipogenic conversion. Though observed, adipogenic conversion in passaged cultures is decreased compared to primary cultures.

Conclusions:

From this work we conclude that: 1) viable preadipocytes exist within primary human liposuctioned fat tissue; 2) they can be successfully isolated from large, exploitable volumes of 'raw' liposuction effluent; and 3) they can be culture-expanded and terminally-differentiated ex-vivo. This work represents the first such evidence that liposuction is a source of viable preadipocytes that may be useful for tissue engineering and gene therapy applications.

Genetic Manipulation of Stromal Cells Isolated from Human Liposuctioned Adipose Tissue and Uses Thereof

A J Katz et al.

Introduction:

Gene therapy technologies are rapidly approaching a developmental stage that will permit more widespread clinical use than previously encountered. Ex-vivo gene therapy, in particular, involves the harvest, isolation, culture and genetic 'engineering' of a patient's (autologous) cells outside of the body. After inserting a specific gene into such culture-expanded autologous cells, the resulting 'genetically engineered' cells are then administered back into the patient for an intended therapeutic effect. One limiting factor to this ex-vivo gene therapy approach, however, is the lack of practical, clinically-useful quantities of autologous tissue/cells for genetic alteration and subsequent protein delivery. Commonly used autologous tissue sources today include bone marrow, skin and blood. The harvest and use of these tissue sources, however, is limited by potential morbidities associated with their removal. For each, a trade-off exists between amounts required for efficient and efficacious clinical utility and the possible physical, physiological and emotional morbidities that may result from the harvest of such amounts. In order to avoid significant morbidities, smaller amounts of autologous tissue are harvested. Starting with fewer cells means that longer time periods are required for the cell-expansion and genetic alteration stages of preparation, thus delaying formal therapy. As an example, such 'preparatory' time delays average around 8 weeks when autologous dermal fibroblasts (skin) are the delivery cell employed. (see enclosed article by Elder, Lotze and Whiteside) Even with this somewhat conservative protocol, the patient is still left with a significantly sized, cosmetically deforming scar at the skin harvest/donor site (10-20 cm2). Moreover, many of these harvest/donor procedures require an operating room with general anesthetic, and are therefore associated with significant risk and expense.

Ideally, what is needed for ex-vivo gene therapy strategies is an autologous tissue source that is abundant, expendable, easy to harvest and which contains cells amenable to culture-expansion, genetic alteration and re-implantation. Adipose tissue (fat) represents an unimagined candidate for such a cell-source as it uniquely meets three of the desired criteria mentioned above. First, fat tissue is abundant on both a societal and individual level. According to a 1995 report by the Institute of Medicine, nearly 60% of the adult population in the United States meets the current definition of clinical obesity. Second, adipose tissue is uniquely expendable. Nearly everyone has enough subcutaneous adipose tissue to donate generous amounts for autologous therapies without any significant biological or anatomical consequences. The same degree of expendability does not exist for bone marrow, blood and skin. Third, fat tissue is extraordinarily easy to obtain (even in large quantities) using the minimally-invasive techniques of liposuction. During the two decades since its introduction, liposuction has continually grown in popularity due in part to refined techniques and instrumentation that make it safer and more reliable. Today, it is the most performed aesthetic operation in the world.

Liposuction was originally intended for the removal of unwanted fat tissue and is still predominately utilized as such. Because fat is a source of several different cell types, however, it also represents an excellent way to harvest autologous tissue for potential tissue engineering and gene therapy applications. Adipose tissue is historically perceived as a static collection of engorged, lipid-filled adipocytes that have hypertrophic but not hyperplastic capabilities. In fact, fat is a surprisingly active and dynamic tissue composed of several different cell types including adipocytes and various stromal cells (including fibroblasts/preadipocytes, pericytes/smooth muscle cells and endothelial cells). And while it was once thought that tissue obtained via liposuction was rendered nonviable due to the trauma inherent in the suction process, it has subsequently been shown by our lab and others that this is simply not the case. Our studies confirm the viability of significant populations of mature adipocytes and stromal cells in liposuction-harvested human adipose tissue. Using our patented device and method, we have been able to successfully culture-expand stromal cells isolated from large

volumes of human liposuctioned adipose tissue(refer to patent #5,786,207, as well as recent invention disclosures). We now present data that demonstrates that human lipo-derived stromal cells are amenable to ex-vivo genetic alteration and capable of subsequent protein expression in-vitro as well as in-vivo following implantation.

Methods:

Human subcutaneous adipose tissue was obtained from elective liposuction procedures with appropriate consent. The raw liposuction effluent (ranging from 100 - 5000mls) was strained in our proprietary device (U.S. patent # 5,786,207) or layered in a modified media bottle to 'refine' (isolate and wash) the actual pieces of adipose tissue from the associated liquid waste consisting of oil, serum, blood, anesthetic solution, etc. The refined fraction of tissue was then subjected to an enzymatic dissociation and filtration process to create a single cell suspension. Stromal cells were separated into a pellet by centrifugation and washed several times by repeated centrifugation and resuspension. Red blood cells were lysed and the remaining cell suspension filtered to remove debris and cell aggregates. Cell number and viability were determined with trypan blue dye exclusion and a hemacytometer. Stromal cells were plated onto standard culture flasks at densities ranging from 20,000 - 100,000 cells/cm2 and grown in DMEM/Ham's F12 media (1:1) with 10% fetal bovine serum and penicillin/streptomycin until they reached confluence. The growth factor bFGF can be added to expedite cell proliferation.

Transduction Studies

For in-vitro transduction studies, expanded cells were exposed to a given viral vector carrying a marker protein (e.g. lacZ or Green Fluorescent Protein) or a bioactive factor (e.g. bFGF or Interleukin 12). Transduction efficiency and protein expression was then determined by staining (lacZ), fluorescent microscopy/flow (GFP) or ELISA (bFGF,IL-12). For in-vivo implantation studies, culture-expanded cells were transduced with an Adenovirus-GFP vector. 48 hrs. after transduction, cells were trypsinized and injected into nude mice in a 'delivery vehicle' containing matrigel and bFGF. Acellular controls were injected in parallel. In-vivo GFP expression was then evaluated by fluorescent microscopic evaluation of frozen-sectioned implants at various timepoints.(please refer to enclosed abstracts for further experimental detail).

Results:

Stromal cells have been repeatedly and routinely isolated and cultured from human liposuction effluent in our lab. Based on an n=20 patients, our techniques yield an average of 319,000 viable stromal cells for every ml of refined liposuctioned adipose tissue digested (range 17,000 - 790,000 viable cells/ml of tissue). In-vitro studies with culture-expanded human lipo-derived stromal cells demonstrate that they are quite amenable to transduction and subsequent protein expression. Using three different viral vectors (retrovirus, adenovirus and herpes simplex virus) we have demonstrated the expression of LacZ and/or GFP by genetically-altered lipo-derived cells. Transduction efficiencies differed depending on the vector used (not surprisingly), revealing >95% efficiency with AdenoV-GFP and HSV-lacZ vectors and only 5-15% efficiency using retrovirus-lacZ (please see photos). More recently we have established stable human lipo-derived cell lines that express either IL-12 or GFP after transduction with retroviral vectors and subsequent selection with antibiotic resistance. Yet other in-vitro studies have demonstrated the successful transduction and expression of bFGF by human lipo-derived cells infected with an HSV-bFGF construct. Levels of bFGF secretion into the media supernatant was measured with an ELISA and revealed supra-basal expression of bFGF 24-72 hours post-transduction.(please refer to abstract: "Basic Fibroblast Growth Factor Gene Therapy Using Human Lipo-Derived Cells") Most, if not all, of the above studies have been repeated at least in duplicate.

Implantation studies have demonstrated the expression of genes inserted into human lipo-derived cells ex-vivo after subsequent implantation in-vivo. More specifically, culture-expanded cells transduced with an adenoviral-GFP vector revealed excellent expression in-vitro 48 hrs. post-transduction as determined by fluorescent microscopy. After subcutaneous implantation into

nude mice, these genetically altered cells continued to express GFP two weeks post-implantation. (please see abstract: "Liposuctioned Fat: A Potential Source of Autologous Cells For Gene Therapy Applications", enclosed)

Conclusions:

Our research demonstrates that stromal cells can be efficiently and routinely isolated from human liposuctioned adipose tissue. These cells can be culture-expanded and are amenable to genetic alteration using various viral-mediated techniques. Furthermore, these cells are capable of expressing genes introduced via these methods both in-vitro and in-vivo after implantation. The proprietary, scientific and commercial significance of this research is embodied by both the cell source (fat tissue) and the manner of tissue harvest (liposuction). Together, they represent a novel and highly exploitable platform technology. The vision of being able to offer future patients novel and valuable autologous therapies based on the removal and 'recycling' of their unwanted, excess fat 'waste' is undeniably appealing to both patient and physician.

ABSTRACT

Many emerging tissue-engineering strategies will benefit from the use of mesodermal-lineage stem cells. Human bone marrow-derived mesenchymal stem cells have been described and have been found to be experimentally useful for these strategies. However, the usefulness of these cells may be limited by several issues including the inherent difficulties in harvesting bone marrow as well as the rarity of the cells and the resultant need for culture expansion using specific sera lots. We hypothesized that adipose tissue, like bone marrow, may also contain a population of mesodermal-lineage stem cells. Since adipose tissue is readily available from humans in large amounts through the process of liposuction, fat tissue may represent a clinically useful population of such cells.

We developed specialized cellular processing techniques to take raw liposuction aspirate from elective cosmetic surgery patients and to obtain a heterogeneous fibroblast-like population of cells. We then found that this fibroblast-like population of cells, when placed in the appropriate media supplementation, was not only able to differentiate into mature adipocytes, but could also differentiate into cells producing bone matrix, cartilaginous matrix, and multinucleated muscle cells. In addition, these freshly harvested fibroblast-like cells from liposuction fat also have telomerase activity, highly suggestive of the stem cell phenotype.

Because human adipose tissue is uniquely expendable, easy to harvest, and contains cells with multi-differentiative potential, we think that human fat may be able to be 'recycled' and may therefore play an important role in future emerging cell-based therapies such as tissue engineering.

INTRODUCTION

Many tissue engineering strategies incorporate the use of biodegradable polymers seeded with some type of cells. The ideal cell with which to seed these tissue engineered constructs depends on the tissue or organ desired. However, several general properties are advantageous, specifically that the cells are 1) autologous, 2) have multi-differentiative potential, 3) can provide a stem population for renewal of the tissue, and 4) are easily obtainable in clinically significant quantities. For engineering mesodermal-derived tissues, bone marrow-derived mesenchymal stem cells have proven experimentally promising. Human bone marrow is a mesodermally derived tissue and appears to contain a population of mesodermal lineage stem cells in addition to hematopoietic stem cells. These cells known as mesenchymal stem cells, can differentiate into fat, bone and cartilage when given the appropriate media supplementation or placed in the appropriate growth conditions.[1] There are, however, problems with this technology. The bone marrow harvest procedure is painful, particularly in the postoperative period and although as much as 20-50 cc of bone marrow can safely be obtained, only a fraction of the harvested cells have the stem/progenitor cell phenotype. It has been estimated that only $1x10^5$ cells in bone marrow are mesenchymal stem cells.[2, 3] Due to the relatively low concentration of stem cells, an in vitro stem cell expansion step is required to obtain clinically useful numbers of cells. This fact prohibits the use of bone marrow-derived mesenchymal stem cells in a 'rapid-turn-around' or bedside harvest and replacement strategy. Furthermore, to maintain these cells in an undifferentiated state, the cumbersome use of specific sera lots is required.[4]

Like bone marrow, adipose tissue is also mesodermally derived. However, new evidence relating to the biology of fat is fundamentally changing our concept of adipose tissue. Fat is no

longer considered a quiescent, static tissue, but appears to have significant endocrine, angiogenic, and structural properties. Based on a new appreciation for this active state and the mesodermal derivation of fat tissue, we hypothesized that adipose tissue may, like bone marrow, also contain a population of cells that can differentiate into multiple cell types.

Fat tissue is not only an active tissue, but also has unique practical qualities that may enhance its use as a potential source of cells for tissue engineering. From the standpoint of using autologous cells for tissue engineering, fat tissue is unmatched by any other tissue, in regard to its availability, expendability, and ease of procurement. Adipose tissue is pathologically abundant in both society and in the average individual. Nearly 60 percent of the adult US population can be classified as being overweight or obese.[5] In addition, adipose tissue has no known physiologic or anatomic morbidities associated with its removal. Furthermore, through liposuction, it is extraordinarily easy to obtain large quantities of fat, even several liters safely and with minimal patient discomfort. Finally, liposuction as a tissue harvest procedure has the secondary gain of improving the body habitus and shape of the patient, which makes it an appealing donor source of cells for the patient.

Our aim was to show that a population of cells could be obtained from liposuctioned fat, which has the potential to differentiate into fat, bone, muscle and cartilage. Furthermore, we sought to determine if this population of cells expresses the putative stem cell marker, telomerase.

MATERIALS AND METHODS

Cell Isolation and Culture

Human adipose tissue was obtained from elective liposuction procedures (n=10) with approval of appropriate institutional review boards (HSPC#98-08-011-02). The fat was then

washed three times with an equal volume of PBS in a separatory funnel, 0.05% Collagenase (Sigma, St Louis, MO) was added to the sample and the mixture was agitated at 37°C for 30 minutes. The solution was then neutralized with DMEM and FBS and the high-density fraction was sedimented after centrifugation at 1,200rpm for 10 min. The pellet was resuspended in DMEM with Erythrocyte Lysing Buffer and agitated at room temperature for 10 minutes. The sample was centrifuged to obtain a pellet, which was then resuspended and filtered, using 100μm nylon mesh. Cells were then plated and cultured (Figure 1).

Cells were cultured in a 37°C incubator with 5% CO_2 in selected media. Culture media was changed twice weekly as indicated. Cells were grown to confluence in a basal media (without specific sera lots), which does not promote cellular differentiation. When confluent, the cells were subjected to media with specific growth supplements (Table 1).

Assessment of Adipogenic Differentiation

Oil Red-O: An assessment of intracellular lipid droplet accumulation was performed using Oil Red-O (Sigma, St. Louis, MO) staining. The culture dishes were fixed with 10% formal calcium prior to staining. Staining was accomplished by washing with 70% ethanol followed by Oil Red-O reagent for 5 minutes. The culture dishes were serially washed and counter-stained with hematoxylin.

Assessment of Osteogenic Differentiation

Von Kossa: Cell fixation was accomplished with 4% paraformaldehyde prior to staining. Culture dishes were rinsed and then coated with 1% silver nitrate solution under ultra-violet light for 45 minutes. After washing with distilled water, they were treated with 3% sodium

thiosulphate, washed with distilled water, and counter stained with van Gieson (acid fuchsin, picric acid) reagent. Staining was qualitatively analyzed under phase microscopy.

Alkaline Phosphatase: Cells were stained according to the insert protocol accompanying the Alakaline Phosphatase kit (Sigma, St. Louis, MO; Procedure 85).

Assessment of Myogenic Differentiation

Screening for the presence of skeletal muscle cells/fibers in cultures was initially performed by light microscopic analysis. Muscle fibers have a unique and characteristic elongated, multinucleated appearance. Further confirmation of the myogenic phenotype was performed by immunohistochemical staining for myosin heavy chain using the monoclonal antibody MF-20 (Developmental Hybridoma Bank) using an avidin-biotin complex method.

In short, cells were rinsed twice with phosphate buffered saline (PBS), fixed for one minute with cold methanol (-20°C), and then rinsed again with PBS. They were then incubated in 0.3% hydrogen peroxide in PBS for 30 minutes, followed by incubation in 10% goat serum (Sigma, St. Louis, MO) in T-PBS (PBS with 0.5% Tween 20) for 30 minutes. After removing the goat serum, cells were incubated for 1 hour in primary antibody (MF-20 hybridoma supernatant). This was followed by three 1-minute rinses with T-PBS and then incubation for 1-hour with goat anti-mouse IgG conjugated to biotin (1:250 dilution in T-PBS; Sigma, St. Louis, MO). This secondary antibody was then removed and the cells rinsed as described above. The cells were then incubated with extravidin peroxidase at 1:250 dilution in T-PBS for 45 minutes (Sigma, St. Louis, MO). After rinsing again, the staining procedure was completed by the addition of diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in PBS with

final concentrations of 0.5mg/ml DAB and 0.015% hydrogen peroxide. The reaction was terminated after several minutes by removing the substrate and adding PBS.

Assessment of Chondrogenic Differentiation

For chondrogenic induction, isolated cells were plated in micromass culture. Briefly, 10 µl of concentrated cell suspension (8 x 10^6 cells/ml) were plated in the center of a well and allowed to attach at 37° C for two hours. Then, the remainder of media was gently added to each well so as not to detach the previously plated cells. Cultures were maintained in a basal media throughout and media was changed twice weekly. Control cultures consisted of cells plated in standard fashion and density. Cultures were followed microscopically and at various time points after plating were stained for the presence of alkaline phosphatase. The presence of sulfated proteoglycans in the matrix was determined by staining with alcian blue at pH 1.

Telomerase Expression

The TRAP assay was performed as previously described with minor modifications.[6] The TRAP-eze telomerase detection kit (Oncor Inc., Gaithersburg, MD) was used to determine telomerase activity. Pelleted cells were dissolved in 100µl of 1x CHAPS buffer, incubated on ice for 30 minutes and centrifuged at 12,000 rpm at 4°C for 30 minutes. The supernatant aliquoted and frozen at -20°C for the TRAP assay and determination of protein concentration. A telomerase positive cell extract (Hela) was used as the positive control and the negative control was a normal human oral fibroblast (NHOF) cell line known to not express telomerase.

The telomerase reaction mixture was created with 2µl of cell lysate (1.0 µg of cellular protein), 48µl of 1x TRAP reaction buffer, 50µM of each deoxyribonucleotide triphosphate,

0.05μg of TS primer end-labeled with 20μCi of [γ-^{32}P] dATP, 1μl primer mix, and 0.4 unit Taq Polymerase. The mixture was incubated at 30°C for 30 minutes and the reaction product amplified by PCR. The PCR products were run in a 12.5% nondenaturing polyacrylamide gel in 1X Tris-borate EDTA for 90 min at 60W. After drying the gel, the signal was determined by autoradiography.

RESULTS

Adipogenic Differentiation

Adipo-derived cells were reproducibly driven toward adipose formation (Figures 2 and 3). Cells grown in adipogenic media began to show evidence of lipid droplet formation, by Oil Red-O staining, as early as 7 days into culture. The formation of lipid droplets then steadily increased and over 80% of cells contained multiple lipid vacuoles by 2 weeks into culture. After this point, the adipocytes became spherical, lost contact with the culture plate and floated to the media surface. In contrast, there was no formation of lipid droplets in the samples which were grown in control media.

Osteogenic Differentiation

Adipo-derived cells which were grown in osteogenic media formed dense, multi-layered nodules composed of spindle shaped cells. These nodules stained positively for alkaline phosphatase as well as for the presence of calcified matrix via von Kossa staining (Figure 4). Adipo-derived cells grown in control media did not show any evidence of bone matrix formation after 6 weeks in culture.

Myogenic Differentiation

Microscopic analysis revealed the presence of discrete 'patches' of large, elongated cells with multiple nuclei in some cultures from several patients. Immunohistochemical staining of these cultures were positive using a monoclonal antibody directed against myosin heavy chain (Figure 6).

Chondrogenic Differentiation

These cultures revealed the development of dense nodules of rounded cells in a dense matrix. The cells stained positively for alkaline phosphatase and nodules stained positive with alcian blue pH1, confirming the presence of sulfated proteoglycans in the matrix (Figure 7). Furthermore, the cells exhibited a rounded morphology typical of chondrocytes.

Telomerase Expression

Cell extracts derived from adult adipose tissue samples were assayed for telomerase activity by the PCR-based TRAP assay (Figure 8). The cell extract derived from immortalized HeLa cells was used as the positive control, which produced the characteristic 6-bp ladder (TTAGGG hexamers), indicating that telomerase was responsible for the reaction. Normal human oral fibroblasts (NHOF) were used as a negative control as this cell line has been established to not contain telomerase activity. Telomerase activity was detected in adipo-derived cells at levels that were qualitatively equivalent to a known positive keratinocyte cell line (NHOK) and bone marrow-derived mesenchymal stem cells.

DISCUSSION

Historically, adipose tissue has been perceived as a collection of quiescent lipid filled adipocytes that contains hypertrophic, but not hyperplastic capability. However recent data confirm the active and dynamic nature of adipose tissue. Adipose tissue, though composed significantly of mature adipocytes, also contains many other cell types including preadipocytes, smooth muscle cells, endothelial cells and fibroblasts.[7] Several studies have shown the ability to digest intact adipose tissue in a variety of animal models producing a heterogeneous cell population.

Embryologically, adipose tissue originates from the mesodermal layer of the developing embryo. It has been suggested that adipocytes arise from the multi-potential stem cell that also gives rise to progenitor cells including bone, muscle, cartilage, and other mesodermal tissues throughout the body. The earliest unipotential cell believed to belong to the adipogenic lineage has been termed the adiploblast and is thought to be derived from mesodermal lineage stem cells. The adiploblast is thought to be committed to the adipogenic lineage and becomes a preadipocyte. Preadipocytes are fibroblast-like cells devoid of intracellular lipid but have the potential to differentiate into mature adipocytes. However, the exact nature and differentiative potential of these cell populations has not been elucidated.

Stem cells are important not only for tissue engineering but for gene therapy applications and for the long term delivery of cells or growth factors for the treatment of a variety of disorders. The concept of a mesodermal-lineage stem cell has existed for several decades. Observations by Friedenstein in the early 1970s revealed a population of cells in the bone

marrow that had the capacity for mesodermal differentiation.[8] Subsequently, Caplan further refined the methods of isolation of a mesodermal-lineage stem cell, or mesenchymal stem cell, based on bone marrow processing and density gradient centrifugation.[4, 9] These cells could then be culture expanded using specific lots of sera. Subsequently, these cells have been shown to exhibit a multi-differentiative potential, including the ability to differentiate into chondrocytes, osteoblasts, adipocytes and myoblasts. The stem cell phenotype was recently confirmed through dilutional cloning.[1] Furthermore, specific cell-surface markers and early transcription factors consistent with lineage -specific differentiation to fat, bone, and cartilage were also defined.

In this study, we found that human mesodermal-lineage cells from fat also exhibited a multi-differentiative potential. We were able to induce osteogenic, chondrogenic, adipogenic, and myogenic differentiation of adipo-derived cells following their mitotic expansion in vitro.

To induce adipogenic differentiation, cAMP agonists and induction agents such as isobutylmethylxanthine and indomethacin were used to supplement the media in conjunction with insulin and glucocorticoids. We found that by 14 days in culture, nearly 80 percent of the primary human cells showed accumulation of lipid droplets by Oil Red-O staining. These cells underwent the typical morphologic changes accompanying adipogenic differentiation, including the early formation of small multifocal vacuoles, which gradually coalesced into larger vacuoles. Coincidentally, the cells appeared to take on a more spherical shape, lose their attachment to the culture flask, and float to the surface of the media.

Osteogenic differentiation occurred with the addition of ascorbic acid and dexamethasone to the culture media. Early evidence of the secretion of mineralized matrix was found after two weeks in vitro. This finding steadily increased over the observation period such that by six weeks in vitro the culture flask was covered with a calcium phosphate matrix.

Many have postulated that the chondrogenic and osteogenic lineages are perhaps two different endpoints along the same continuum.[10-15] From this perspective, our findings are consistent with previous work. When cultured in micromass conditions to simulate the hypoxic environment characterstic of cartilage, human adipo-derived cells developed into dense nodules of cells and matrix that stained positive for alkaline phosphatase and alcian blue at pH 1. Again, the expression of alkaline phosphatase is consistent with osteochondrogenic development. Positive staining with alcian blue of nodules demonstrated the presence of sulfated proteoglycans in the matrix, another finding supportive of the chondrogenic phenotype.

The explanation for the development of skeletal muscle fibers in some of our cultures is more difficult to interpret. These cells were not induced with specific media, rather, they were observed empirically in several cultures nourished only with basal media. Although the myogenic phenotype is derived from the mesodermal embryonic layer along with bone, fat, cartilage, tendon, etc. and has been obtained from mesenchymal stem cells, the sporadic appearance of these muscle cells from fat may have alternative explanations. One possibility is that some fat tissue specimens were "contaminated" with myoblasts (myofiber progenitors) which survived culture expansion and subsequently differentiated into myofibers. However, when performing liposuction, it would be difficult to enter the fascia with a blunt tipped liposuction cannula. Furthermore, myogenic differentiation has been noted in tissue cultures from several patients.[16] An alternative hypothesis is that myoblasts unknowingly reside in subcutaneous fat depots in the natural state. Though normally 'dormant', they may function in wound healing and repair when nearby muscle is damaged. This is supported by Ferrari et al, who demonstrated the regeneration of muscle by progenitors existing within the stroma of bone marrow - another "fatty" depot.[17]

While the exhibition of multi-differentiative capability represents persuasive evidence to the existence of a stem cell from human liposuctioned fat, it is not conclusive. It is possible that liposuctioned fat contains a population of progenitor cells for a variety of committed progenitor cells for a variety mesodermal lineages. We have yet to definitively clone a population of cells and show evidence of multipotentiality from that specific population. It is also possible that there is a dedifferentiation phenomenon attributable to a population of cells in liposuction fat. Literature has shown that preadipocytes or mature adipocytes can "dedifferentiate" into a fibroblastic cell population. It is possible that this dedifferentiation phenomenon can be extrapolated to a resetting of the differentiative potential to the cell, encompassing not only adipogenic differentiation, but also other mesodermal lineages as well. This behavior would however, be relatively unique for nature. It is also possible that contamination of the liposuction aspirate with a stem cell source from skin, fascia, nerve and muscle, may also occur. However, given our technique of liposuction, it is very unlikely that the skin or fascial cavity surrounding the muscles could be violated during the procedure. In general, liposuction takes place on the deep surface of the fat well away from the skin to avoid contour irregularities. In addition the rigidity of the muscular fascia and the blunt nature of the liposuction cannula tips makes it very unlikely that a cannula tip could be placed through the fascia sampling muscle tissue. Another explanation for the existence of stem or progenitor cells in liposuction fat is that these cells represent circulating cells that could perhaps originate from the bone marrow. Although this is possible, the magnitude of differentiation evident in vitro suggests that the fibroblast like cells that we obtain from liposuction fat are in a much higher concentration that would be explained by the theoretic number of cells that might be found in the circulating blood. In addition, all of the tissue obtained from our liposuction procedures were obtained when a vasoconstrictive

compound, epinephrine, was applied in the subcutaneous fat minimizing blood contamination. Another exploration is that the cells are actually pericytes or smooth muscle cells. Pericytes are known to surround small blood vessels and have been shown to exhibit osteogenic differentiation in vitro and in vivo.[18, 19] However, a more complex explanation would be required to explain multi-lineage differentiation. We are currently in the process of determining whether pericytes or smooth muscle cells might be involved in the multi-lineage differentiation process. However, irrespective of the exact nature of the stem or progenitor cell, the practical advantages of using fat derived cells for cell based tissue therapies is alluring.

To further support the existence of a stem-like population of cells in adipose tissue, we evaluated the presence of telomerase expression in this heterogeneous cell population. Stem cells are able to maintain their telomeric length through the use of the enzyme telomerase. Telomerase activity has recently been shown to be present in bone marrow-derived mesenchymal stem cells, bone marrow-derived hematopoietic cells and embryonic stem cells.[1, 20] In addition, telomerase activity is found in cells with malignant potential and those cells that have a rapid proliferative potential such as normal human oral keratinocytes. We found that unpassaged, primary fibroblast-like cells from liposuctioned fat expressed telomerase whereas human foreskin fibroblasts do not. While the expression of telomerase does not conclusively show the presence of a stem-like cell in liposuction fat, it is highly suggestive.

The future of engineering mesodermal-derived tissues from autologous cells is promising. The development of these tissue engineering strategies will likely require a readily available source of donor cells. These cells are ideally autologous, available in large quantities, and have minimal morbidity associated with their harvest. While marrow-derived mesenchymal stem cells represent the only known source of these cells, we believe that cells from

liposuctioned fat may be a more practical alternative. Irrespective of the ontogeny of these cells, the sheer volume and availability makes them an attractive source of cells for future cell based therapies. Their autologous nature, multipotentiality, ease of procurement, and the large volumes available are significant advantages to cells harvested by liposuction. Experiments are currently ongoing to determine: (1) through dilutional cloning, if an ***adipo-derived stem cell*** definitively exists in human liposuctioned fat tissue, (2) if so, what is its ontologic similarity to marrow-derived mesenchymal stem cells and (3) the in vivo utility of these cells for tissue engineering.

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Tables and Legends

Table 1

Media Conditions	Additives
Control	DMEM, 10% FBS, 1% ABAM
Adipogenic	DMEM, 100nM dexamethasone, 100nM insulin, 0.5 mM IBMX, 100mM indomethacin, and 1% ABAM
Osteogenic	DMEM, 10% FBS, 7% Horse Serum, 5%Chick embryo extract, 100nM dexamethasone, 10mM ßGP, 50µM Ascorbic acid, and 1% ABAM
Chondrogenic	Micromass technique using DMEM, Ham F12, 10% FBS, and 1% penicillin/streptomycin

DMEM = Dulbecco's modified essential medium, FBS = fetal bovine serum, ABAM = penicillin sodium 100U/cc + streptomycin sulfate 100 µg/ml + amphotericin B 0.25 µg/ml, IBMX = isobutylmethylxanthine, ßGP = beta-glycerolphosphate

Table 1. Culture conditions for control media, adipogenic, osteogenic, and chondrogenic differentiation in vitro.

1. A method of isolating stromal cells from adipose tissue, said method comprising

a) obtaining adipose tissue from an animal,

b) dissociating said adipose tissue into a suspension comprising aggregated cells and separated cells comprising stromal cells and red blood cells within a supernatant,

c) separating said cells from said supernatant by centrifuging said suspension until said cells form a pellet overlaid by said supernatant,

d) removing said supernatant from said pellet and resuspending said pellet in a physiologically compatible suspension fluid,

e) lysing said red blood cells to produce debris,

f) separating said isolated stromal cells from said debris and said aggregated cells by filtering said suspension, and

g) plating said isolated stromal cells onto a culture substrate.

2. The method of claim 1, wherein said stromal cells comprise mesenchymal stem cells (MSCs).

3. The method of claim 1 or 2, wherein said animal is a human.

4. The method of any of claims 1-3, wherein said adipose tissue is obtained by obtaining raw liposuction effluent from an animal, said effluent comprising adipose tissue and separating said adipose tissue from the remainder of said effluent,

5. The method of any of claims 1-4, wherein said adipose tissue is separated by density differential and/or centrifugation from the remainder of said effluent.

6. The method of any of claims 1-5, wherein said separated adipose tissue is dissociated into said suspension by enzymatic digestion or mechanical dissociation.

7. The method of any of claims 1-6, wherein said adipose tissue is separated from the remainder of said effluent and dissociated into said suspension by:

one or more
a) providing a system for dissociating tissue having a housing unit having one or more compartments located therein having a plurality of fluid

communicating means between the interior of said compartments and the interior of said housing unit, one or more inlet ports that provide communication between the exterior environment and said interior of at least one of said compartments and provide means for introducing solutions into said interior of at least one of said compartments, one of said inlet ports having a filter and providing a means for filtered air to enter said housing unit, one of said compartments having a means for engaging a rotating agitating, or centrifugation mechanism, a means for rotating, agitating, or centrifuging said compartment, a heat source, said housing unit having at least one aperture exit, a means for controlling the exit of effluent through said aperture, via gravity, positive, or negative pressure, a first receptacle sealably engaging said housing unit, and a second receptacle wherein said second receptacle is centrifuge-ready and sealably engages said housing unit;

b) inserting tissue to be dissociated into a first of said compartments via one of said inlet ports;

c) inserting a washing solution into a second of said compartments via another of said inlet ports;

d) permitting said washing solution to descend through said plurality of fluid communicating means between said interior of said first of said compartments and said interior of said housing unit, through said pores providing for fluid communication between said interior of said second of said compartments and said interior of said housing unit, and into said second of said compartments where it interacts with said tissue inserted therein;

e) placing said means for controlling the exit of effluent through said aperture in an open position such that said effluent exits the housing unit and is collected in said first receptacle;

f) placing said means for controlling the exit of effluent through said aperture in a closed position and disengaging said first receptacle and sealably engaging said second receptacle;

g) inserting a proteolytic enzyme, detergent, or chemical-digestive solution into said housing unit;

h) heating said proteolytic enzyme solution using said heat source while agitating, rotating, or centrifuging said second of said compartments within said housing unit using said means for agitating, rotating or centrifuging said second compartment and thereby facilitating tissue dissociation;

i) placing said means for controlling the exit of effluent through said aperture in an open position such that effluent exits said housing unit and is collected in said second receptacle;

j) collecting the cell suspension resulting from tissue dissociation in said second receptacle.

8. The method of claim 1, wherein steps d and e are repeated one or more times.

9. The method of any of claims 1-8, wherein said red blood cells are lysed by resuspending said pellet within a hypotonic fluid.

10. The method of any of claims 1-9, wherein the viability of said stromal cells is assessed.

11. The method of claim 10, wherein said viability is assessed by trypan blue exclusion.

12. The method of claim 1, wherein said stromal cells are plated at a density of about 20,000 cells/cm^2.

13. The method of claim 1, wherein said stromal cells are plated at a density of about 40,000 cells/cm^2.

14. The method of claim 1, wherein said stromal cells are plated at a density of about 60,000 cells/cm^2.

15. The method of claim 1, wherein said stromal cells are plated at a density of about 80,000 cells/cm^2.

16. The method of claim 1, wherein said stromal cells are plated at a density of about 100,000 cells/cm^2.

17. The method of any of claims 1-16, wherein said plated stromal cells are expanded by culturing them in a tissue culture medium.

18. The method of claim 17, wherein said medium comprises DMEM and/or Ham's F12 media.

19. The method of claim 17 or 18, wherein said medium comprises serum.

20. The method of any of claims 17-19, wherein said medium comprises at least one antibiotic.

21. A method of obtaining a genetically-modified stromal cell comprising:

a) isolating a stromal cell from adipose tissue in accordance with any of claims 1-20, and

b) exposing said stromal cell to a gene transfer vector comprising a nucleic acid including at least one transgene, whereby said nucleic acid is introduced into said stromal cell under conditions whereby said at least one transgene is expressed within said stromal cell.

22. The method of claim 21, wherein said gene transfer vector comprises a plasmid.

23. The method of claim 21, wherein said gene transfer vector comprises a virus.

24. The method of claim 23, wherein said virus is an adenovirus, an adeno-associated virus, a retrovirus, or a herpesvirus.

25. The method of any of claims 21-24, wherein at least one transgene encodes a protein conferring resistance to a toxin.

26. A method of obtaining a transformed stromal cell line comprising:

a) obtaining a genetically-modified stromal cell according to the method of claim 25,

b) expanding said cell within a medium comprising said toxin, and

c) passaging cells exhibiting resistance to said toxin.

27. A method of delivering a transgene to an animal comprising:

a) obtaining a genetically-modified stromal cell in accordance with claims 21-25, and

b) introducing said cell into said animal, such that said transgene is expressed in vivo.

28. A method of differentiating adipose-derived stem cells, said method comprising:

a) isolating and expanding stromal cells from adipose tissue in accordance with claim 17, and

b) culturing said expanded cells in a serum-free morphogenic medium,

c) monitoring said cells to identify morphological differentiation.

29. The method of claim 28, wherein said morphogenic medium is an adipogenic medium and said cells are monitored identify adipogenic differentiation.

30. The method of claim 29, wherein said adipogenic medium comprises a glucocorticoid, insulin, a compound which elevates intracellular levels of cAMP, and/or a compound which inhibits degradation of cAMP.

31. The method of claim 30, wherein said glucocorticoid is isobutyl-methylxanthine, dexamethasone, hydrocortisone, or cortisone.

32. The method of claim 30, wherein said compound which elevates intracellular levels of cAMP is dibutyryl-cAMP, 8-CPT-cAMP (8-(4)chlorophenylthio)-adenosine 3', 5' cyclic monophosphate; 8-bromo-cAMP; dioctanoyl-cAMP or Forskolin.

33. The method of claim 30, wherein said substance which inhibits degradation of cAMP is a phosphodiesterase inhibitor.

34. The method of claim 33, wherein said phosphodiesterase inhibitor is methyl isobutylxanthine, theophylline, caffeine, or indomethacin.

35. The method of any of claims 29-34 wherein said adipogenic differentiation is monitored by observing the rounding of cells and the accumulation of intracytoplasmic lipid droplets.

36. The method of claim 35, wherein said intracytoplasmic lipid droplets are detected by oil red O staining.

37. The method of claim 28, wherein said morphogenic medium is an osteogenic medium and said cells are monitored identify osteogenic differentiation.

38. The method of claim 37, wherein said osteogenic medium comprises beta-gycerophosphate, ascorbic acid and/or dexamethasone.

39. The method of claim 38, wherein said osteogenic differentiation is monitored by assaying for calcium in the extracellular matrix.

40. The method of claim 38 or 39, wherein said osteogenic differentiation is monitored by observing cells stained positively for alkaline phosphatase and von Kossa's staining method.

41. The method of claim 28, wherein said morphogenic medium is an chondrogenic medium and said cells are monitored identify controgenic differentiation.

42. The method of claim 41, wherein said chondrogenic medium simulates a hypoxic environment.

43. The method of claim 42, wherein said hypoxic environment is simulated by culturing said cells at high density.

44. The method of claims 42 or 43, wherein said cells are cultured at about 8 million cells/ml.

45. The method of any of claims 42-44, wherein said cells are cultured using micromass culture technique.

46. The method of any of claims 42-45, wherein said chondrogenic differentiation is monitored by assaying for sulfated proteoglycans in the extracellular matrix.

47. The method of any of claims 42-46, wherein said chondrogenic differentiation is monitored by observing cells stained positively for alkaline phophatase and alcian blue at pH of about 1.

48. The method of claim 28, wherein said morphogenic medium is an myogenic medium and said cells are monitored identify myogenic differentiation.

49. The method of claim 48 wherein said myogenic differentiation is monitored by assaying for myosin heavy chain.

50. The method of claim 49 wherein said myogenic myosin heavy chain is assayed by immunohistochemical techniques.

51. The method of any of claims 28-50, wherein said stromal cells are mesenchymal stems cells (MSCs).